

**A. Status of the claims**

Claim 1 is amended to recite a nucleic acid sequence that hybridizes under stringent conditions to SEQ ID NO:2, a reference nucleic acid sequence. The hybridization conditions are recited in the claim. This amendment adds no new matter. Support for this amendment can be found, *e.g.*, in the specification on page 21, lines 9-11 and in originally filed claim 8.

Claims 6 is amended to recite stringent hybridization conditions. This amendment adds no new matter. Support for this amendment can be found, *e.g.*, in the specification on page 21, lines 9-11.

Claim 9 is amended to recite moderately stringent hybridization conditions. This amendment adds no new matter. Support for this amendment can be found, *e.g.*, in the specification on page 21, lines 14-21.

Claim 6 was further amended to clarify the relationship between amplification primers and degenerate PCR primers, *e.g.*, they hybridize under stringent conditions to the same sequence. This amendment adds no new matter.

**B. Rejections under 35 U.S.C. §112, second paragraph**

Claims 6, and 8-9 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite for reciting the phrases “stringent hybridization conditions,” “stringent conditions,” and “moderately stringent hybridization conditions.” Office Action at page 3. The Office Action alleges one of skill would not be able to determine the metes and bounds of the claims. In order to expedite prosecution, Applicants have amended claims 6 and 9 to recite hybridization conditions and canceled claim 8 without prejudice. Applicants therefore respectfully request the rejection be withdrawn with respect to claims 6 and 9.

Claim 6 is also rejected as being vague because allegedly, it is not clear that limitations are being added to the claimed nucleic acid. The Office Action also alleges the meanings of “same sequence” and “degenerate primer sets” are unclear.

Applicants respectfully traverse the rejection. With regard to the addition of limitations, claim 6 limits independent claim 1 by requiring that the encoded polypeptide include two specific amino acid sequences: MLGQQQQ and KKDRRSR. Applicants respectfully point out that those amino acid sequences are the seven C-terminal and seven N-terminal sequence of the p33ING2 protein (SEQ ID NO:1). Thus, while claim 1 is directed to a nucleic acid which encodes a protein with 70% identity to p33ING2, claim 6 is directed to a protein with 70% identity to p33ING2, with the caveat that the 14 specifically listed amino acids are among those identical amino acids.

In order to expedite prosecution, Applicants have amended claim 6 to clarify that the amplification primers and the degenerate primers both hybridize the same sequence. In addition, the specification provides ample description of codon degeneracy at page 16, lines 19-35. Based on the disclosure, those of skill in the art would be able to design degenerate primers that encode a specific amino acid sequence.

### **C. Rejections under 35 U.S.C. §112, first paragraph**

#### *1. Introduction*

Claims 1-2, 5-9, and 20-21 are rejected as allegedly claiming subject matter that is not enabled by the specification. The rejection states that nucleic acids encoding polypeptide variants are not enabled. Furthermore, the Examiner is apparently concerned about inoperable embodiments. *See, e.g.,* Office Action, pages 6-7.

As identified in the Patent Office and the Federal Circuit, whether undue experimentation is required by one skilled in the art to practice an invention is determined by considering factors such as the amount of guidance presented in the application, the state of the prior art, and the presence of working examples. *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int. 1985); *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). As described in *Wands*, a “considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which experimentation should precede.” *Wands*, 8 USPQ2d at 1404 (quoting *In re Jackson*, 217 USPQ 804 (Bd. Pat. App. & Int. 1982).

The claims now specify hybridization conditions, as well as conserved reference sequences to which the claimed nucleic acids must hybridize. Hybridization methods for the identification of nucleic acids are also well known to those of skill in molecular biology. These elements therefore provide adequate guidance for routine identification of the nucleic acids of the invention. In addition, claimed functional characteristics of the proteins encoded by the claimed nucleic acids would allow one of skill in the art to identify operable embodiments and exclude inoperable embodiments. Finally, Applicants clearly meet the PTO guidelines for enablement, which set forth the standard for the scope of enablement when a large number of possible embodiments exists. Thus, undue experimentation is not required to practice the claimed invention.

*2. The claimed reference sequences provide a meaningful structural feature that allows one of skill to identify the claimed sequences without undue experimentation.*

The rejection alleges that the specification provides enablement only for a nucleic acid encoding a p33ING2 polypeptide comprising an amino acid sequence of SEQ ID NO:1 or for an isolated nucleic acid comprising SEQ ID NO:2. However, the claims recite both functional and structural characteristics of the p33ING2 nucleic acids of the invention. The present application also provides functional assays for identification of nucleic acids encoding p33ING2 polypeptides of the invention, without undue experimentation. The assays and examples of the specification, together with standard methodology known to those of skill in the art, therefore provide adequate guidance for identifying claimed nucleic acids that encode the p33ING2 polypeptides of the invention.

The assertion of undue experimentation appears to be based on an assumption that enablement requires the description of each and every nucleic acid that could be covered in the invention. As noted below, such a requirement is not consistent with the patent laws. Indeed, it is well settled in the biotechnology art that routine screening of even large numbers of samples is not undue experimentation when a probability of success exists. *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). Using the

conditions set forth in the claims and specification and routine methodology, any competent laboratory technician in a molecular biology laboratory could isolate and prepare appropriate constructs, transform cells, and identify those nucleic acids that encode a p33ING2 protein of the invention. As set forth in MPEP § 2164.08, a rejection for undue breadth is inappropriate where “one of skill could readily determine any one of the claimed embodiments.” In the present case, one of skill, given the conserved amino acid and nucleotide sequences and the specified hybridization conditions, could easily screen for other nucleic acid and protein molecules that fall within the scope of the claims.

The present invention describes a family of nucleic acids encoding tumor suppressor polypeptides which functionally are p33ING2 polypeptides and which structurally hybridize to reference nucleic acids.

At the time of the present invention, identification of nucleic acids having the functional and structural characteristics described above was well within the means of one of skill of the art, without undue experimentation. The present specification provides working examples and discloses standard techniques known to those of skill in the art, for the identification of functional p33ING2 polypeptides such as that exemplified by SEQ ID NO:1. In addition, one of skill in the art could use standard hybridization and PCR assays to identify nucleic acids encoding the polypeptides of the invention (*see, e.g.,* specification, pages 20-21).

Finally, functional assays to identify p33ING2 polypeptides of the invention are known to those of skill in the art and are disclosed in the specification. For example, the specification describes methods of assaying for changes in cell proliferation by expressing p33ING2 constructs in transformed cells. Exemplified assays of p33ING2 function include loss of ability to grow in soft agar (page 40-41), contact inhibition of cell growth (page 41-42), dependence on serum for growth (page 42), decrease in tumor specific markers (page 42-43), decreased ability to invade matrigel (page 43-44), increased levels of apoptosis (page 44), and ability to cause cell cycle arrest (page 44-45).

The assays described in the specification, coupled with methodology well known to those of skill in the art, therefore demonstrate that screening for nucleic acids which encode p33ING2 polypeptides having the structural and functional characteristics described above is routine. *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). Applicants therefore respectfully request that the rejection be withdrawn.

*3. One of skill in the art could readily determine any one of the claimed nucleic acids.*

Finally, regarding the issue of enablement for nucleic acids, where a large number of possible embodiments exist, the PTO has provided express guidelines for examination. As set forth in the MPEP § 2164.08, a rejection of claims such as those in the present application for undue breadth is inappropriate where one of skill could readily determine any one of the claimed embodiments.

This standard is further explained in the “Training Materials for Examining Patent Applications with respect to 35 U.S.C. § 112, first paragraph – Enablement Chemical/Biotechnological Applications,” section III.A.2.b.i(c). In the guidelines, the PTO specifically answers the question regarding scope of a nucleic acid composition claim (e.g., in the present case, a nucleic acid encoding a p33ING2 protein) left open by the Federal Circuit in *In re Deuel*, 34 USPQ2d 1210, 1216 (Fed. Cir. 1995). The claims at issue in *Deuel* were directed to any DNA encoding a specific amino acid sequence. Thus, a great number of nucleic acids were within the scope of the claims. In fact, the number was so great that a listing of all possible DNAs encoding the protein was a practical impossibility.

In the guidelines, the PTO addressed this issue, explaining that “even though a listing of all possible DNAs which encode a given protein is a practical impossibility due to the enormous number of such nucleic acids, any particular sequence can be written by one of skill given the disclosure and the sequence can be ordered from a company which synthesizes DNA.” In this manner, one of skill in the art can readily

determine any one of the embodiments. The PTO concluded that scope rejections such as the one hypothesized in *Deuel* should not be advanced.

In the present application, one of skill in the art has only to identify nucleic acids that either (1) hybridize under specified conditions to the conserved reference nucleotide sequence of SEQ ID NO:2; or (2) are amplified by the primers that hybridize under specified conditions to the conserved reference sequences, using techniques described in the specification or known to those of skill in the art. Although many such nucleic acids are possible, one of skill can readily determine, one by one, any particular p33ING2 encoding nucleic acid, without undue experimentation. For example, nucleic acid screening, hybridization, and PCR techniques are described in the specification and the art, as described above. Furthermore, one of skill can use the assays described above to test the functionality of the protein encoded by the nucleic acid of interest and easily determine if it falls within the scope of the claims. Thus, in the present application the skilled artisan can readily, with only routine experimentation, make and test any particular p33ING2 encoding nucleic acid.

The specification, combined with the state of the prior art, thus provides a number of different assays demonstrating that any experimentation required to identify nucleic acids encoding p33ING2 proteins is not undue. *In re Wands*, 8 USPQ 1400 (Fed. Cir. 1988). Applicants respectfully request that the rejection be withdrawn.

#### **D. Rejections under 35 U.S.C. §102 (a) and (b)**

Claims 1-9 are rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Shimada *et al.*, *Cytogenet. Cell. Genet.* **83**:232-235 (1998). Because the month of publication of Shimada *et al.* was not known, the Office Action also states the rejection will be maintained the under §102(a) if necessary.

Claims 1-2, 6-9, 20-21 are rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Bonaldo *et al.* (Genbank Database, Accession No. BF523624, as allegedly published in *Genome Research*, Vol. 6, (9), 1996). The Examiner cited both the Genome research article by Bonaldo *et al.* and the Genbank submission.

Applicants respectfully traverse the rejection because the Office Action has mischaracterized the references as prior art. Shimada *et al.* was not publicly available as a printed publication until after the earliest priority date of the present application. Bonaldo *et al.* is not prior art because it is not an enabling reference, (e.g., it does not disclose the claimed nucleic acid sequence). Like Shimada *et al.*, the cited GenBank submissions were not publicly available until after the earliest effective filing date of the present application and are thus not prior art.

*1. The earliest effective filing date of the application is February 26, 1999 and that date should be used to determine what is prior art.*

Applicants respectfully bring to the Examiner's attention a claim for priority to the provisional application, U.S.S.N. 60/121,891, which has a filing date of February 26, 1999. (Specification at page 1, lines 7-9.) Thus, the earliest effective filing date of the Application is February 26, 1999.

*2. Shimada et al. is not prior art under either 35 U.S.C. §102(a) or (b).*

Shimada *et al.* was not published before the earliest effective filing date of the application and is therefore not prior art.

a. Under 35 U.S.C. §102 (a) or (b), the date of publication is determined by the date of public accessibility.

Under 35 U.S.C. §102(b) a person "shall be entitled to a patent unless the invention was...described in a printed publication in this or a foreign country...more than one year before the date of application for patent in the United States." Courts have defined the phrase 'printed publication' as follows: "The statutory phrase 'printed publication' has been interpreted to mean that before the critical date the reference must have been sufficiently accessible to the public interested in the art; dissemination and public accessibility are the keys to the legal determination whether a prior art reference was 'published'." *Constant v. Advanced Micro-Devices* 848 F.2d 1560 1568 (Fed. Cir.

1988). The same definition of printed publication applies to analysis of prior art under 35 U.S.C. §102(a).

In order to be considered prior art under §102(b), a printed publication must have been publicly available more than one year before the earliest effective filing date of the application. In order to be considered prior art under §102(a), a printed publication must have been publicly available before the invention date of the Applicant. In the U.S., with out other evidence, the earliest effective filing date for filing the application is presumed to be the constructive invention date. Thus, a reference published after February 26, 1999 is not prior art for the current application under either 35 U.S.C. §102(a) or (b).

b. Shimada *et al.* was not publicly available until well after the earliest effective filing date of the application.

The Office Action alleges that Shimada *et al.* was published in 1998 and at the same time acknowledges that the exact month of publication is unknown. Office Action at page 8. Using the alleged 1998 publication date, the Office Action rejects claims 1-9 under either 35 U.S.C. §102(a) or (b).

Applicants respectfully traverse the rejection. Under 35 U.S.C. §102(a) or (b), the publication date of Shimada *et al.* is determined by the date of public accessibility of the reference. A publication date recited by a publisher does not control the outcome.

According to the Federal Circuit, evidence can be submitted to prove the date a reference became publicly accessible and therefore “published.” *Constant*, 848 F.2d at 1569. Applicants submit a date stamped copy of the Shimada *et al.* reference, received by The University of California at Berkeley on April 23, 1999, as proof of the date of public accessibility, and therefore publication of the reference. The date is well after the earliest effective filing date of the present application. Thus, the Shimada *et al.* reference is not prior art under either 35 U.S.C. §102(a) or (b).

The Office Action also included a copy of a GenBank listing for the protein disclosed in the Shimada *et al.* reference. The date of submission and thus, public



accessibility of the GenBank Accession, is March 18, 1999, also well after the earliest effective filing date of the present application. Thus, the GenBank submission is not prior art under either 35 U.S.C. §102(a) or (b).

2. *Neither the Bonaldo et al. Genome Research article nor the Bonaldo et al. GenBank Accession is prior art under 35 U.S.C. §102(b).*

In order to be considered prior art under 35 U.S.C. §102(b), a publication must be both enabling and publicly available more than one year before the earliest priority date of the application.

a. The Bonaldo et al. Genome Research article is not an enabling disclosure, and thus, is not prior art under 35 U.S.C. §102(b).

As stated by the Court of Customs and Patent Appeals, the proper test of an enabling description in a publication cited under §102(b) is:

whether one skilled in the art to which the invention pertains could take the description of the invention in the printed publication and combine it with his own knowledge of the particular art and from this combination be put in possession of the invention on which a patent is sought. Unless this conditions prevails, the description in the printed publication is inadequate as a statutory bar to patentability under section 102(b). *In re LeGrice*, 301 F.2d 929, 939 (C.C.P.A. 1962).

For an invention such as an isolated protein or DNA molecule, a printed publication must include an enabling disclosure, (e.g., the amino acid or nucleic acid sequences). A reference that does not disclose either the amino acid sequence or the nucleic acid sequence does not provide a disclosure that enables one of skill in the art to make and use the nucleic acid or its encoded protein.

The Bonaldo et al. Genome Research article discloses a **method** to make normalized cDNA libraries. The reference does not disclose the sequence of any gene or protein found in the libraries, including those of the claimed invention. Because the

sequences of the p33ING2 nucleic acid and protein are not disclosed in the Bonaldo *et al.* Genome Research article, the reference is not enabling and therefore, is not prior art.

b. The Bonaldo *et al.* Genbank Accession is not prior art under 35 U.S.C. §102(b).

The Examiner included a GenBank submission from The Bonaldo *et al.* that cites the 1996 Bonaldo *et al.* Genome Research article. As previously discussed, that article disclosed only the methods used to make libraries. The nucleic acid sequences found in the libraries were sequenced and disclosed at a later date. The Bonaldo *et al.* Genbank submission has a date of 11-DEC-2000 (Top line of GenBank listing), well after February 29, 1999, the earliest priority date of the present application. Because the sequence of the Bonaldo *et al.* Genbank Accession was not disclosed until after the earliest effective filing date of the present application, the Bonaldo *et al.* Genbank Accession is not properly cited as prior art for the present application.

Because the cited references are not prior art, Applicants respectfully request that the rejections under 35 U.S.C. §102(a) and (b) be withdrawn.

**E. Rejections under 35 U.S.C. §103(a)**

Claims 1-9, 20-21 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Shimada *et al.* The Office Action alleges it would have been *prima facie* obvious to one of ordinary skill in the art to include an expression vector and host cell with the teachings of Shimada *et al.*

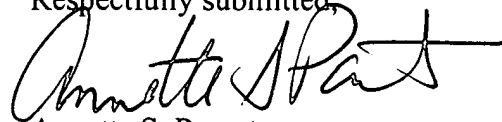
Applicants respectfully traverse. As described above, Shimada *et al.* is not prior art because it was not publicly accessible until well after the earliest effective filing date of the application. Thus, the rejection under 35 U.S.C. §103(a) was made improperly and should be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Annette S. Parent", with a stylized flourish at the end.

Annette S. Parent  
Reg. No. 42,058

TOWNSEND and TOWNSEND and CREW LLP  
Two Embarcadero Center, 8<sup>th</sup> Floor  
San Francisco, California 94111-3834  
Tel: (415) 576-0200  
Fax: (415) 576-0300  
ASP/BLK  
SF 1346844 v1



APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (Once amended) An isolated nucleic acid encoding a tumor suppressor polypeptide p33ING2[, wherein the polypeptide has greater than 70% amino acid sequence identity to a polypeptide comprising an amino acid sequence of SEQ ID NO:1] that specifically hybridizes under stringent conditions, wherein the hybridization reaction is incubated at 42°C in a solution comprising 50% formamide, 5x SSC, and 1% SDS and washed at 65°C in a solution comprising 0.2x SSC and 0.1% SDS, to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2.
  
6. (Once amended) The isolated nucleic acid of claim 1, wherein the nucleic acid is amplified by primers that selectively hybridize under stringent hybridization conditions, wherein the hybridization reaction is incubated at 42°C in a solution comprising 50% formamide, 5x SSC, and 1% SDS and washed at 65°C in a solution comprising 0.2x SSC and 0.1% SDS; to [the same] a nucleotide sequence, wherein the nucleotide sequence also hybridizes under stringent conditions to [as] degenerate primer sets encoding amino acid sequences selected from the group consisting of SEQ ID NO:3 (MLGQQQQ and SEQ ID NO:4 (KKDRRSR).
  
9. (Once amended) The isolated nucleic acid of claim 1, wherein said nucleic acid selectively hybridizes under moderately stringent hybridization conditions, wherein the hybridization reaction is incubated at 37°C in a solution comprising 40% formamide, 1 M NaCl, and 1% SDS and washed at 45°C in a solution comprising 1x SSC, to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2.

APPENDIX B

CLAIMS CURRENTLY UNDER EXAMINATION

1. (Once amended) An isolated nucleic acid encoding a tumor suppressor polypeptide p33ING2 that specifically hybridizes under stringent conditions, wherein the hybridization reaction is incubated at 42°C in a solution comprising 50% formamide, 5x SSC, and 1% SDS and washed at 65°C in a solution comprising 0.2x SSC and 0.1% SDS, to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2.
3. (As filed) The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a polypeptide comprising an amino acid sequence of SEQ ID NO:1.
4. (As filed) The isolated nucleic acid sequence of claim 1, wherein the nucleic acid comprises a nucleotide sequence of SEQ ID NO:2.
5. (As filed) The isolated nucleic acid of claim 1, wherein the nucleic acid is from a human.
6. (Once amended) The isolated nucleic acid of claim 1, wherein the nucleic acid is amplified by primers that selectively hybridize under stringent hybridization conditions, wherein the hybridization reaction is incubated at 42°C in a solution comprising 50% formamide, 5x SSC, and 1% SDS and washed at 65°C in a solution comprising 0.2x SSC and 0.1% SDS; to a nucleotide sequence, wherein the nucleotide sequence also hybridizes under stringent conditions to degenerate primer sets encoding amino acid sequences selected from the group consisting of SEQ ID NO:3 (MLGQQQQ) and SEQ ID NO:4 (KKDRRSR).

7. (As filed) The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a polypeptide having a molecular weight of about 28 kDa to about 38 kDa.

9. (Once amended) The isolated nucleic acid of claim 1, wherein said nucleic acid selectively hybridizes under moderately stringent hybridization conditions, wherein the hybridization reaction is incubated at 37°C in a solution comprising 40% formamide, 1 M NaCl, and 1% SDS and washed at 45°C in a solution comprising 1x SSC, to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2.

20. (As filed) An expression vector comprising the nucleic acid of claim 1.

21. (As filed) A host cell transfected with the vector of claim 20.

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# Cloning of a novel gene (ING1L) homologous to ING1, a candidate tumor suppressor

Y. Shimada, A. Saito, M. Suzuki, E. Takahashi and M. Horie

Otsuka GEN Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima (Japan)

**Abstract.** The ING1 gene encodes p33<sup>ING1</sup>, a putative tumor suppressor for neuroblastomas and breast cancers, which has been shown to cooperate with p53 in controlling cell proliferation. We have isolated a novel human gene, ING1L, that potentially encodes a PHD-type zinc-finger protein highly homologous to p33<sup>ING1</sup>. Fluorescence in situ hybridization and radiation-hybrid analyses assigned ING1L to human chromosome 4. Both ING1 and ING1L are expressed in a variety of

human tissues, but we found ING1L expression to be significantly more pronounced in tumors from several colon-cancer patients than in normal colon tissues excised at the same surgical sites. Although the significance of this observation with respect to carcinogenesis remains to be established, the data suggest that ING1L might be involved in colon cancers through interference with signal(s) transmitted through p53 and p33<sup>ING1</sup>.

The ING1 gene was identified as a candidate tumor suppressor through a strategy based on subtractive hybridization and subsequent selection of transforming gene fragments termed "genetic suppressor elements" (Garkavtsev et al., 1996). Expression of ING1 is up-regulated in senescent fibroblast cells (Garkavtsev and Riabowol, 1997); its over-expression inhibits cell growth by arresting cells in the G<sub>1</sub> phase of the cell cycle (Garkavtsev et al., 1996) and enhances the extent of apoptosis in the absence of survival factors (Helbing et al., 1997).

ING1 encodes a nuclear protein, p33<sup>ING1</sup>, that contains a zinc-finger motif (Garkavtsev et al., 1996). p33<sup>ING1</sup> increases p53-dependent transcriptional activation of the p21 gene (CDKN1A alias WAF1) (Garkavtsev et al., 1998), whose product is known to mediate p53-dependent growth arrest (reviewed by Levine, 1997). This growth-suppressive effect requires normal activity of both genes (ING1 and TP53) (Garkavtsev et al., 1998). The likelihood that ING1 cooperates with

TP53 is supported by the ability of their products to associate physically (Garkavtsev et al., 1998).

Zinc-finger motifs comprise several structural subfamilies (Schwabe and Klug, 1994), and most of them are thought to participate in recognition of macromolecules such as DNA, RNA and protein. The PHD-type zinc-finger domain was first identified in two closely related plant homeodomain-containing proteins, HAT3.1 and HOX1A (Schindler et al., 1993). The PHD finger, a C4HC3-type motif spanning 50–80 amino acid residues, has since been found in a number of chromatin-mediated transcriptional regulators from a variety of sources (reviewed by Aasland et al., 1995). We report here the isolation of a novel human gene of the PHD-finger family, ING1L, which bears striking sequence homology to ING1, a candidate tumor suppressor gene. We also provide preliminary data with respect to the expression of ING1L in normal human tissues and in colon tumors.

## Materials and methods

### Isolation of ING1L cDNA and genomic DNA clones

From the private Otsuka cDNA data base (available from the GENOTK web site: <http://genotk.genome.ad.jp:8010/>) we identified a human fetal brain cDNA, GEN-146F11, as a candidate for encoding a molecule homologous to the putative tumor suppressor p33<sup>ING1</sup>. The 446-bp insert of GEN-146F11, which encoded 99 carboxyl-terminal amino acids, was used as a probe for screening a  $\lambda$ gt10 human fetal brain cDNA library (Clontech). We obtained cDNAs containing the 5'-region of the novel gene designated ING1L (GenBank accession no. AB012853).

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Request reprints from Dr. Masato Horie, Otsuka GEN Research Institute, Otsuka Pharmaceutical Co., Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192 (Japan); telephone: 81-886-65-2888; fax: 81-886-37-1035; email: mhorie@otsuka.genome.ad.jp

CGGCC  
ATGTT  
M LAGCCG  
S RGACAT  
D MAAGGA  
K ECGTC1  
R LATACA  
I QCACTY  
H SGATTY  
D SCGTG/  
R DAAGA/  
K KTCAC/  
S PTCTT/  
S YTCATY  
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TCCAAGCTTGAAGCTGAGGCGCCGCGCGCGCCG	31
LOGGCGGTGCATGTCGCGCTGCTGGAATCGCGAGCGCGCGCGCGCGAGTTCGCGAGG	91
ATGTTTAGTTGGGACGACGACGACCAACTGTACTCGTGGCGCGCGCTCTGTACCGGGAGCGG	151
H L G Q Q Q Q Q L Y S S A A L L P G E R	20
AGCCGGCTGCTCACCTGCTACGTGCAGGACTACCTTGAGTGCCTGGAGTCGCTGCCCCAC	211
S R L L T C Y V Q D Y L E C V E S L P H	40
GACATGCGACGAGGAACGTGTCTGTGCTGCGAGAGCTGGACAAATAATATCAAGAAACGTTA	271
D M Q R N V S V L R E L D N K Y Q E T L	60
AAGGAAATTGATGATGCTCTACGAAAAATATAAGAAAAAGATGATTTAAACCGAGAAGAAA	331
K E I D D V Y E K Y K K E D D L N Q K K	80
GTCTACAGCAGCTTCTCCAGAGAGCACTAATTAATAGTCAAGAATTTGGGAGATGAAAAA	391
R L Q Q Q L L Q R A L I N S Q E L G D E K	100
ATACAGATTGTTACACAAATGCTCGAATTTGGTGGAAAAATCGGGCAAGACAAATGGAGTTA	451
I Q I V T Q M L E L V E N R A R Q M E L	120
CACCTACAGTGTTCCTCAAGATCTCTGCTGAAAGTGAACGAGCCTCAGATAAAGCAAGATG	514
H S Q C F Q D P A E S E R A S D K A K M	140
GATTCCAGCCAACCGAGAAAGATCTTCAAGAAGACCCCGCAGCGACCGACCAAGTGAAGC	571
D S S Q P E R S S R R P R R Q R T S E S	160
CGTGATTTATGTACATGGCAAAATGGGATTTGAAGACTGTGATGATCAGCCACCTAAAGAA	631
R D L C H M A N G I E D C D D Q P K E E	180
AAGAAATCCAAGTCAGCAAGAAAAAGAAACGCTCCAAGGCCAAGCAGGAAAGGGAAGCT	694
K K S K S A K K K K R S K A K Q E R E A	200
TCACCTGTTGAGTTTGCAATAGATCCTAATGAACCTACATACTGCTTATGCAACCAAGTG	751
S P V E F A I D P N E P T Y C L C N Q V	220
TCTTATGGGGAGATGATAGGATGTGACAATGAACAGTGTCCAATTGAATGGTTTCACTTT	811
S Y G E M I G C D N E Q C P I E W F H F	240
TCATGTGTTTCACTTACCTATAAAACCAAGGGGAAATGGTATTGCCCAAGTGCAGGGGA	871
S C V S L T Y K P K G K W Y C P K C R G	260
GATAATGAGAAAAAATGGACAAAAGTACTGAAAAAGACAAAAAGGATAGAAGATCGAGG	931
D N E K T M D K S T E K T K K D R R S R	280
TAGTAAAGGCCATCCACATTTTAAAGGGTTATTTGACTATTATATAATCCGTTTGCTTTC	991
AAGAAATGTTTGGGGTAAATGCAATAAGACTATGCAATAATTATTAATCATTGATTAA	1051
TGGTGTATTAAAGTGTGTGACTTTG	1070

**Fig. 1.** Nucleotide and predicted amino acid sequences of human ING1L. The 1078 nucleotides of total sequence and the translated 280 amino acid residues open reading frame are indicated. Nucleotide and amino acid positions are numbered on right, and predicted amino acids are shown in standard single-letter symbols below each codon. The in-frame stop codon in the 5'-untranslated region is marked with dots; a putative polyadenylation signal (ATTTAA) is underlined.

Screening of the human BAC library (Research Genetics) was carried out according to the manufacturer's procedure using primers designed to amplify nucleotides 759 to 949 of ING1L, 5'-GGGAGATGATAGGATGTGAC-3' (P1) and 5'-TGTGGATGGCCTTTACTACC-3' (P2).

#### Direct R-banding fluorescence in situ hybridization (FISH)

A BAC clone corresponding to ING1L was mapped to chromosome spreads by direct R-banding FISH, a technique based on FISH combined with replicated prometaphase R-bands (Takahashi et al., 1990; 1991). Repetitive sequences present in the BAC were sequestered by a 5-fold excess of human cot-1 DNA (Gibco BRL) as described by Lichter et al. (1990). Signals were captured by CytoVision (Applied Imaging).

### Radiation-hybrid mapping

Mapping was carried out using the Genebridge 4 Radiation-Hybrid Panel (Research Genetics) according to the manufacturer's procedure, using primers P1 and P3 (5'-GCATAGTCTTATGCATTACCC-3') designed to amplify nucleotides 759 to 1026 of the INGL1 gene. The amplification profile consisted of 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

INGIL	NLQQQQQQLYSSAALLTGRSRMLTCYVQDYLCVPSLPHQRRHYVVLN	50
p33 <sup>INO1</sup>	NLSFANGGLELNVN-YV-DYLDIESLSPQQRNVSLNR	38
INGIL	ELDMKYQETLKEKEDDYVYKYNEDLQKQQLQRLALISQELGDER	100
p33 <sup>INO1</sup>	SDAKYVORICKECYEYFSTSDAKKRNMLKCYORALISQELGDFE	88
INGIL	IQIVQKELVEVHNKQMLISQCCQDPAS-SERASDKKRNDSPP----	145
p33 <sup>INO1</sup>	IQIVQKELVEVHNKQVDSVVELSAQQSGDTGVGNSQVADRPQDA	138
INGIL	-----ERSSNRPRQFSSSNLCHMANGTICDDQFPKKKRSKAKKKR	190
p33 <sup>INO1</sup>	VAQSDKFPSSSRROSNFNRSSNASSNHDDQASGDTKKKATSKKRN	188
INGIL	RSKAKEREASPFVETIDPNEPTYCLCHNOVSYGEMTICGDNFCPTIEUHF	240
p33 <sup>INO1</sup>	RSKAKEREASPDLDIDPNEPTYCLCHNOVSYGEMTICGDNFCPTIEUHF	238
INGIL	SCVQLNFKPKGKWKYCPKRCGHEKTHDKSLEKRNDRSR	280
p33 <sup>INO1</sup>	SCVQLNFKPKGKWKYCPKRCGHEKTHDKALESKKPRATNR	279

**Fig. 2.** Alignment of deduced amino acid sequences of ING1L with p33<sup>ING1</sup>. Conserved amino acid residues are indicated by the black background. The PHD-type zinc-finger motif is indicated by a shaded bar above this domain; cysteine and histidine residues conserved among PHD-type zinc-finger proteins are marked with asterisks. The amino acid sequence of p33<sup>ING1</sup> was derived from GenBank (accession number AF044076).

### Northern-blot analysis

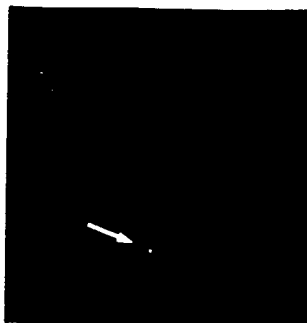
Expression of the ING1L and ING1 genes was evaluated using human multiple tissue Northern blots I and IV (Clontech), as well as Northern Territory™ Human Tumor Panel Blots I-V and the Human Colon Tumor Blot (Invitrogen) according to the manufacturers' procedures. GEN-146F11, human ING1 and human  $\beta$ -actin cDNAs were used as probes. These membranes were prehybridized for 2 h and then hybridized with the probes for 20 h at 65°C in a mixture of 10% dextran sulfate, 0.05% pyrophosphoric acid, 50 mM Tris-HCl (pH 7.5), 0.9 M NaCl, 2  $\times$  Denhardt's solution, and sodium dodecylsulfate (SDS) containing 100  $\mu$ g/ml denatured salmon sperm DNA. Hybridized blots were washed twice with 2  $\times$  standard sodium citrate (SSC), 0.1% SDS at room temperature for 5 min, and then twice with 0.1  $\times$  SSC, 0.1% SDS at 65°C for 15 min.

## Results and discussion

### Cloning of *ING1L* cDNA

To obtain novel genes potentially involved in carcinogenesis, we searched the private Otsuka cDNA data base for sequences bearing homology with putative or defined oncogenes and tumor suppressor genes. This database had been constructed by sequencing cDNAs randomly selected from human placental, aortal and fetal-brain cDNA libraries. We obtained a clone (GEN-146F11) potentially encoding a molecule related to the candidate tumor suppressor ING1, and designated the deduced product of the gene represented by GEN-146F11 as ING1L (for "ING1-like molecule"). The full-length ING1L cDNA contained an open reading frame of 840 bp, encoding a deduced protein of 280 amino acids (Fig. 1) with a predicted molecular weight of 32.8 kDa.

When we compared the deduced amino acid sequence of ING1L with p33<sup>ING1</sup> (Fig. 2), the gene products showed 58.9% identity; nucleotide identity between the genes themselves was 60.0% (data not shown). A PHD-type zinc-finger motif was present in the carboxyl-terminal halves of both ING1L (amino acid residues 213–260) and p33<sup>ING1</sup>.



**Fig. 3.** Chromosome assignment of *ING1L*, as determined by FISH on R-banded human chromosomes. The arrow indicates twin signals on chromosome 4q35.1.

### Chromosomal localization

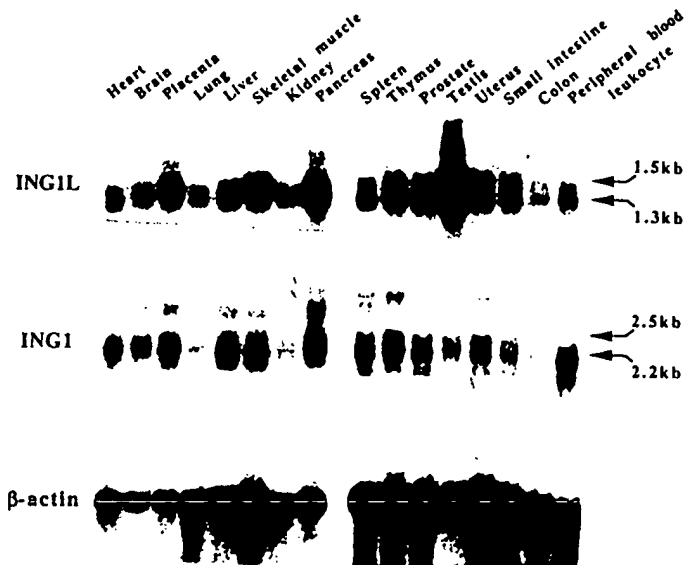
We determined the chromosome location of *ING1L* by direct R-banding FISH (Fig. 3). Twin signals were observed on the q35.1 band of chromosome 4; no signal was observed at any other site. A more precise localization using the Genebridge 4 Radiation-Hybrid Panel (Research Genetics) revealed that *ING1L* lay distal to the WI5831 marker locus on chromosome 4. Despite the striking similarity in their sequences, the *ING1L* and *ING1* genes are not on the same chromosome, *ING1* has been assigned to 13q34 (Garkavtsev et al., 1997; Zeremski et al., 1997).

### Expression of *ING1L* mRNA in human tissues

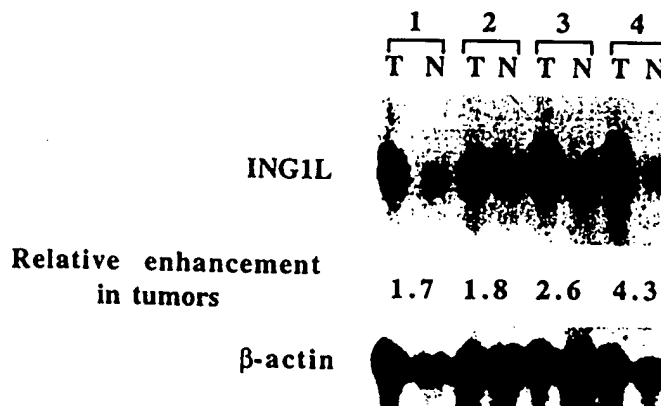
Northern-blot analysis revealed ubiquitous expression of *ING1L* (Fig. 4) in the form of two major bands (1.5 kb and 1.3 kb). The *ING1* probe also detected two major bands (2.5 kb and 2.2 kb) in all tissues examined.

Since expression of *p33<sup>ING1</sup>* is known to be reduced in several breast cancer cell lines (Garkavtsev et al., 1996), we examined expression of *ING1L* in 20 tumors derived from different tissues (data not shown). We found that the novel gene was expressed at a significantly higher level in all colon cancers examined than in normal tissues obtained at the same surgical sites (Fig. 5, our unpublished data). Until the physiological function(s) of *ING1L* can be established, we are unable to account for the stronger expression of the gene in some normal tissues (e.g., in testis) than in others. However, its dramatic increase over normal levels in neoplastic colon is a salient and remarkable finding, and its significance to this common type of cancer and molecular mechanism(s) underlying enhanced expression of *ING1L* in colon tumors need to be investigated.

The presence of a PHD-finger motif suggests that *ING1L* may function as a regulator of transcription. As the amino acid sequences around this motif are highly conserved between *ING1L* and *p33<sup>ING1</sup>* (see Fig. 2), they might compete for the same macromolecule(s). Physical and biological interactions have been demonstrated between *p33<sup>ING1</sup>* and *p53* (Garkavtsev et al., 1998); therefore we consider *p53* to be a good candidate for interaction with *ING1L*. It is of particular interest that the



**Fig. 4.** Expression of the *ING1L* and *ING1* genes as determined by Northern blotting in various human tissues. Human multiple-tissue Northern blots I and IV (Clontech) were hybridized with <sup>32</sup>P-labeled *ING1L*, *ING1* and  $\beta$ -actin probes. The *ING1L*, *ING1* and  $\beta$ -actin hybridizations were autoradiographed respectively after 18 h, 18 h and 35 min at  $-80^{\circ}\text{C}$ .



**Fig. 5.** Increased expression of the *ING1L* gene in human colon tumors as determined by Northern blotting. Northern Territory™ Human Colon Tumor Blots (Invitrogen) were hybridized with <sup>32</sup>P-labeled *ING1L* and  $\beta$ -actin probes, and then the blots were autoradiographed after 72 h and 40 min, respectively, at  $-80^{\circ}\text{C}$ . Samples of total RNA [20  $\mu\text{g}$ ] from tumor (T) and normal (N) tissues from each of the following donors were loaded in adjacent lanes: donor 1, a 37-year-old male patient with adenocarcinoma; donor 2, a 59-year-old male patient with adenocarcinoma; donor 3, a 33-year-old male patient with differentiated adenocarcinoma; donor 4, a 56-year-old male patient with poorly to moderately differentiated carcinoma. Relative enhancement in tumors was calculated as the increase in tumors over the base line expression in corresponding normal tissues after normalization of the values according to the levels of  $\beta$ -actin mRNA expression.

INGIL gene was over-expressed in the colon tumors we examined, because p53 abnormalities occur frequently in this type of carcinoma (Baker et al., 1989). We intend to search for proteins that bind with INGIL, and to investigate the function of this protein and the consequences of its increase within colon tissues. Such information may provide deeper insight into progression of tumors in the colon.

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